

***SMOC-2* suppression in Diffuse Large B-Cell Lymphoma may confer resistance to  
rituximab**

Honors Thesis  
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**Abstract**

Rituximab is a genetically constructed chimeric mouse-human monoclonal IgG1 kappa antibody that recognizes the CD20 antigen. It has been the top selling oncology drug for nearly a decade. Rituximab was approved for medical use in 1997 and since then, it has improved outcomes in all B-cell malignancies. However, about 40% of patients relapse after initially responding to rituximab combined with cyclophosphamide, doxorubicin, vincristine, and prednisone (R-CHOP). It is therefore important to understand any potential mechanisms of resistance in order to improve patient survival through better therapeutic choices. Since rituximab's mechanisms of action are challenging to study in non-human models, our group used *in vitro* studies linking genotype to phenotype via Genome-Wide Association study (GWAS) and identified a Single Nucleotide Polymorphism (SNP) in *SMOC-2*, which encodes an extracellular matrix protein, that might be involved in rituximab sensitivity. In order to determine whether the expression of *SMOC-2* affects rituximab sensitivity, malignant B cells with a *SMOC-2* knockdown were tested in an *in vitro* Complement Dependent Cytotoxicity (CDC) rituximab killing assay. After performing CDC assays on several Diffuse Large B-Cell Lymphoma (DLBCL) *SMOC-2* knockdown cell lines, the HBL-1 cell line exhibited resistance to rituximab. However, western blot analysis yielded inconclusive evidence about the knockdown of *SMOC-2*, since the *SMOC-2* band could not be confidently identified. Understanding the mechanisms of intrinsic and acquired resistance to drugs in cancer therapy may enable the use of patient genotypes to determine which anti-CD20 would be most effective or allow interventions to restore rituximab sensitivity in patients.

## Introduction

Immunotherapies combat cancer by utilizing the body's own immune system. By activating the immune system to specifically target cancer cells, many of the side effects of conventional chemotherapies due to the nonspecific killing of healthy cells, such as hair loss, are avoided. The most common non-Hodgkin lymphoma (NHL) is Diffuse large B-cell lymphoma (DLBCL), which comprises approximately 30% of all new diagnoses.<sup>1</sup> The median age of those presenting is mid-60s. The cyclophosphamide, doxorubicin, vincristine, and prednisone (CHOP) chemotherapy regimen has been the foundation of treatment since its development in the 1970s.<sup>2</sup> CHOP has remained the standard of care since attempts to improve outcomes with more intensive chemotherapy regimens failed to demonstrate any additional benefit.<sup>3</sup> In most cases today, rituximab is used to treat DLBCL in combination with CHOP chemotherapy (R-CHOP). In one study, R-CHOP, given to elderly patients with newly diagnosed DLBCL for eight cycles, significantly increased the level of complete response, decreased the rates of treatment failure and relapse, and improved event-free and overall survival as compared with standard CHOP alone.<sup>4</sup>

Rituximab is a chimeric mouse-human monoclonal antibody consisting of a human IgG1 Fc portion, a human kappa constant region, and a murine variable region which recognizes human CD20.<sup>5</sup> CD20 is a 33 to 36 kDa non-glycosylated phosphoprotein expressed on the cell membrane of mature B cells. Since conventional chemotherapeutic drugs target and kill dividing cells, the side effects of chemotherapy involve healthy body tissues where cells are continually growing and dividing, such as hair, bone marrow, skin, etc. Normal cells can still replace the dead cells or repair the healthy cells that are damaged by chemotherapy once treatment ceases. The CD20 receptor is only expressed by normal B cells but is absent on other normal cell types, such as precursor B cells, dendritic cells, and plasma cells, in a healthy individual. More importantly, 95% of B-cell

NHLs and other B-cell malignancies also express CD-20, which makes it an ideal therapeutic target.<sup>6</sup> CD-20 antibodies only target cells where the CD-20 receptor is expressed, i.e., solely B-cells. Therefore, the classic side effects of chemotherapy are avoided with rituximab. Interestingly, the biological function of CD20 is still unclear, although some evidence suggests a role in  $\text{Ca}^{2+}$  ion influx and homeostasis.<sup>7</sup> CD20 has no known natural ligand, and CD20 mutant mice have a nearly normal phenotype.<sup>8</sup> Once bound to B cells, rituximab induces lysis through several possible mechanisms: induced apoptosis (programmed cell death), antibody-dependent cell-mediated cytotoxicity (ADCC), and complement-dependent cytotoxicity (CDC). The apoptosis of B cells can be initiated simply by the binding of rituximab. In ADCC, an immune response is triggered by the presence of antibodies covering the target cell. Once the antigen is bound to the B cell, the antibody's Fc region is exposed and will bind its receptor on the Natural Killer (NK) cell to form an immune synapse. A lytic signal is then delivered to the target cell by the effector cell, resulting in the target cell's death. In CDC, the binding of C1q to the antibody triggers the complement cascade, which leads to the formation of the membrane attack complex (MAC), causing subsequent cell lysis (Figure 1). While 90% of patients respond to rituximab and chemotherapy via the mechanisms described above, around 30-40% of those 90% relapse.<sup>9</sup> In order to improve rituximab resistant patient survival through better clinical choices, it is important to understand any potential mechanism of resistance.<sup>10</sup> Despite widespread clinical use, the mechanisms by which tumor cells resist rituximab-mediated damage remain unclear.

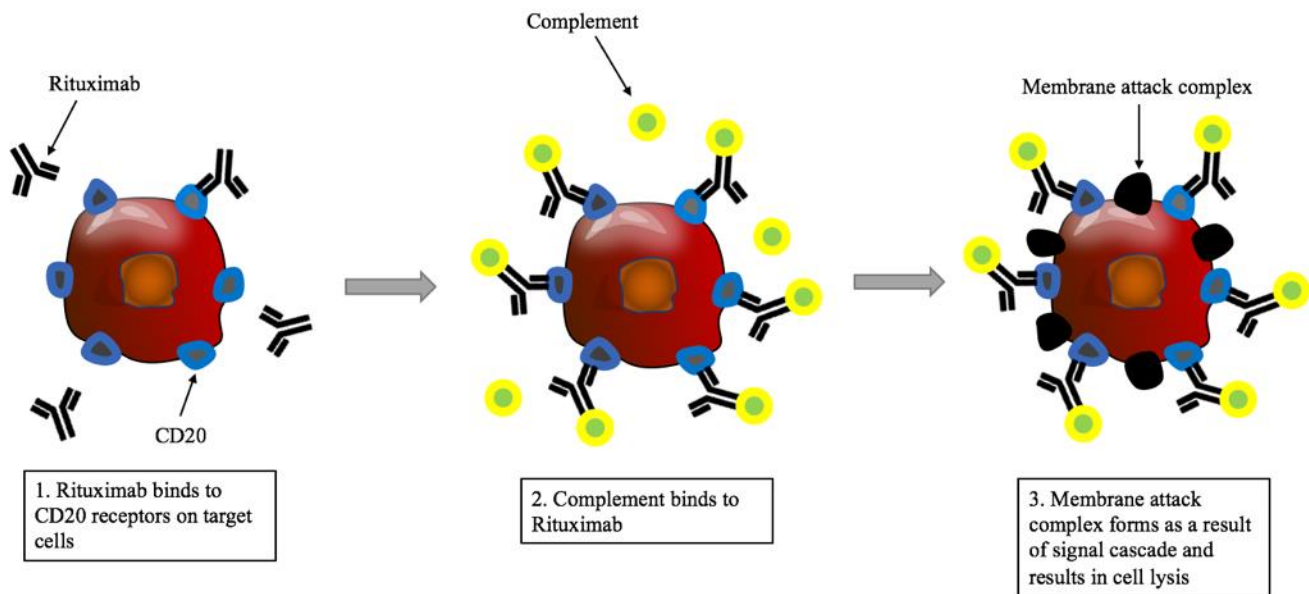


Figure 1. CDC Mechanism of Action

The established definition of rituximab resistance is the lack of response or the progression within 6 months of treatment to a rituximab-containing regimen.<sup>11</sup> Continued disease progression during administration of rituximab is the most clearly defined form of resistance. Individuals who experience tumor shrinkage may have some level of rituximab sensitivity but can still be classified as “resistant” if they do not achieve either a partial or complete response. Various pathways of tumor resistance have been elucidated with conventional chemotherapeutic agents. In some cases, these discoveries have translated into specific interventions intended to prevent or overcome tumor resistance.<sup>11</sup> Surprisingly, little is known about the mechanisms which facilitate rituximab resistance in the treatment of B-cell NHL.<sup>11</sup> The exact mechanism(s) of rituximab’s action in patients, either as a single drug or combined with chemotherapy, remains subject to great uncertainty.

Potential mechanisms of tumor resistance have been described in each of the major pathways of rituximab action: apoptosis induction, ADCC, and CDC.<sup>6</sup> It has been difficult to link the exact involvement of these mechanisms to rituximab resistance. Low levels of apoptosis in tumor cells can be triggered by rituximab binding directly to CD20 receptors.<sup>6</sup> Alterations in the apoptotic pathway signaling could therefore lead to cells becoming resistant to rituximab. Rituximab-resistant cell lines have been produced through repeated exposure to the antibody. These cell lines show resistance to apoptosis and lack sensitivity to multiple cytotoxic chemotherapeutic agents, including rituximab. Numerous variations have been described for pro- and anti-apoptotic regulators in these rituximab-resistant cell lines.<sup>12</sup> The nuclear factor-kappaB (NFkB) pathway is specifically overactivated, leading to increased expression of anti-apoptotic proteins from the Bcl-2 family. By simply exposing these clones to inhibitors of the survival pathways *in vitro*, they can be resensitized to rituximab.<sup>12</sup>

While ADCC is known to be a major contributor underlying the effectiveness of rituximab, the specific pathways by which tumor cells fail to respond to ADCC are unknown. Conformational alterations in the CD20/rituximab complex located in the cell membrane may mediate the recognition and association of bound antibody by effector cells, such as NK cells, macrophages, etc. For example, statins, which are involved in cholesterol synthesis inhibition, impaired rituximab-mediated ADCC *in vitro*, signifying that changes to the lipid raft could lead to ADCC resistance.<sup>13</sup> The patient *FCGR3A* genotype has been repeatedly implicated in rituximab-mediated ADCC. Polymorphisms present in this Fc receptor may affect the affinity of effector cells for rituximab. NHL patients possessing low affinity (158F/F) FcγRIIIa convey lower rates of response and lower progression-free survival when treated with rituximab.<sup>14</sup> ADCC may also be impaired by serum complement activation. C3b, a complement protein, seems to inhibit NK-driven ADCC.

C3b depletion, however, restores ADCC activity in *in vitro* models and improves the survival of rituximab-treated mice in a syngeneic murine lymphoma model.<sup>15</sup> Thus, complement depletion may actually enhance rituximab efficacy by bolstering the efficiency of ADCC. On the other hand, complement depletion seems to have the opposite effect in CDC and may potentially mediate rituximab resistance.

Since CDC also plays a major role in the mechanism of rituximab, a major focus of research into rituximab resistance has been the complement pathway. Tumor cells can block the activation of complement proteins via membrane complement-regulatory proteins (mCRP), which include CD59, CD46, and CD55. These inhibitory proteins disrupt either the assembly of the membrane attack complex or the complement cascade and are widely expressed in nearly all types of cancer, which may confer protection against CDC.<sup>16</sup> Some rituximab-resistant cell lines have been described as expressing high levels of mCRP due to selective pressure from repeated exposure to rituximab.<sup>17</sup> If blocking antibodies neutralize mCRP, the efficacy of rituximab is greatly enhanced.<sup>18</sup> However, mCRP functions in an essential regulatory role in shielding normal cells from uncontrolled complement-mediated cytotoxicity, thus restricting the clinical application of mCRP blockade. Currently, no clinical agents are capable of specifically blocking cancer-related mCRP while maintaining mCRP protection of normal cells. However, it has been observed that the synergy between fludarabine, a chemotherapy medication used to treat leukemia and lymphoma, and rituximab seems to partially mediate the ability of fludarabine to downregulate CD55, which increases susceptibility to rituximab-mediated CDC.<sup>19</sup> Exposure to rituximab may also deplete the complement protein stores. Therefore, rituximab “resistance” might be somewhat mediated by the depletion of the essential effector molecules. Klepfish et al. infused rituximab-resistant Chronic Lymphocytic Leukemia (CLL) patients with plasma, complete with abundant

complement proteins, in combination with doses of rituximab. This approach triggered “a rapid and dramatic clinical response in all patients,” supporting the hypothesis that complement depletion plays a clinically significant role in rituximab resistance.<sup>20</sup> Although complement depletion seems to improve rituximab killing via ADCC, it may also play a role in rituximab resistance in CDC. These contrasting phenomena must be better elucidated with further studies.

One apparent mechanism of rituximab resistance is loss of the target antigen, CD20. The possibility of complete CD20 loss following rituximab therapy appears unlikely. However, more subtle changes in CD20 expression have been implicated in rituximab resistance. Some rituximab-resistant cell lines showed decreased expression of CD20 at both the pre- and post-translational levels. This reduced CD20 expression led to reorganization of the lipid raft and downstream signaling changes, suggesting that the effect of CD20 expression on rituximab resistance is more complex than basic antibody–antigen ratios.<sup>21</sup> Only recently has antigenic modulation of CD20 been demonstrated as a possible resistance mechanism. Beers et al. demonstrated the internalization of CD20 by CLL and mantle cell lymphoma cells exposed to rituximab. Since follicular NHL was fairly resistant to CD20 internalization, this may explain its greater clinical responsiveness to rituximab.<sup>22</sup> Initial reports of acquired CD20 mutations were published when C-terminal deletion mutations of the CD20 gene were identified in a subset of patient tumor samples with NHL.<sup>23</sup> The mean fluorescent intensity of CD20 was decreased because of these mutations, most likely affecting the binding of antibodies, which plays a role in rituximab resistance. Another distinct mechanism for CD20 loss in rituximab-resistant malignancies has been designated as trogocytosis or “shaving.”<sup>24</sup> Rituximab/CD20 complexes can be separated from the surface of B cells by monocytes via the Fc receptor pathway. In other words, the antibody/CD20 complexes are “shaved” from the cell surface instead of being kept exposed for targeted cell death. Trogocytosis

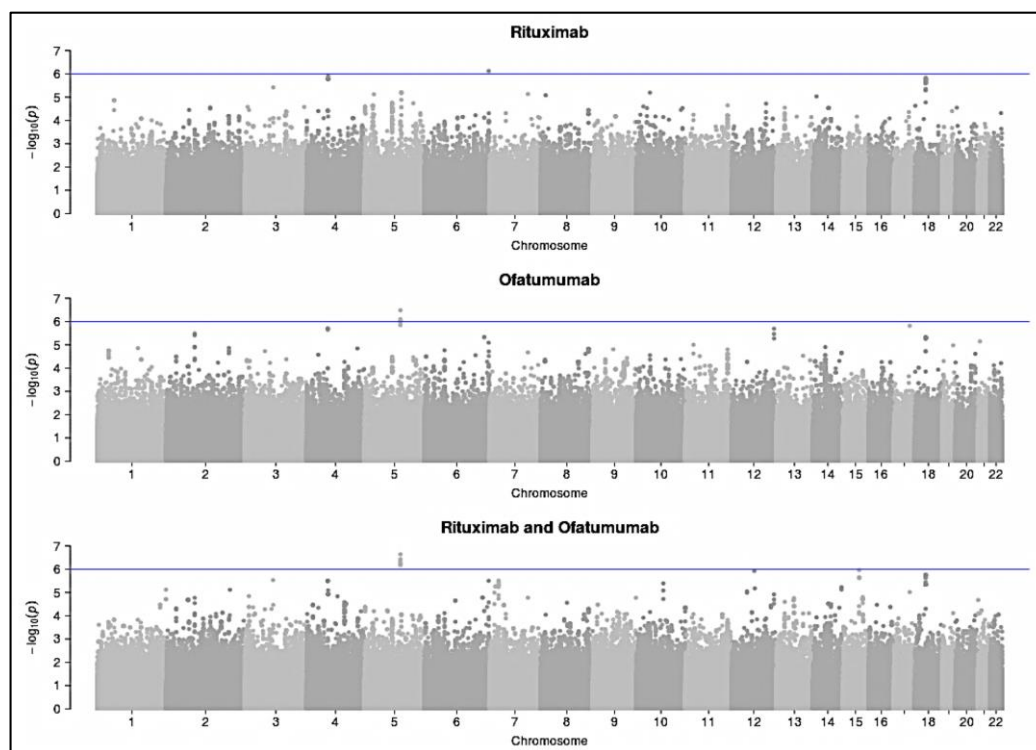


can result in antigen loss and thus rituximab resistance. In the same study that demonstrated this, it was also found that intravenous immune globulin (IVIG) may block this shaving reaction, confirming the idea that it is Fc-receptor-mediated.<sup>25</sup>

Our laboratory has used several genetic approaches including association, linkage, and gene expression to find important genetic factors that mediate monoclonal antibody sensitivity and discovered a role for rituximab drug sensitivity linked to the *CBLB* and *SMOC-2* genes.<sup>26</sup> For *CBLB*, the heritability of rituximab and ofatumumab responses were first investigated in multigenerational families in order to ensure that the phenotypes were heritable and therefore able to be mapped by linkage mapping. Our multitiered linkage analysis strategy, utilizing publicly available genotype and gene expression data, indicated *CBLB* as a gene of interest. We then demonstrated that *CBLB* knockdown leads to rituximab resistance by affecting the localization of CD20 and the susceptibility to CDC.<sup>26</sup> In a similar study, publicly available genotype data was used to analyze associations between loci/genes and drug response. Three different genome-wide association studies (GWAS) were performed: two association analyses representing each drug individually and one association analyses which mutually modelled both drug responses as a vector. Genotypic and phenotypic data from 486 unrelated individuals were used in each model. Genotypic data and cell lines from these individuals were obtained from an unrelated cholesterol GWAS.<sup>27</sup> Phenotypic data were acquired after cells from these individuals were grown in culture and subjected to CDC assays. Finally, rituximab responses were linked to SNPs and their p-values were plotted against chromosomal location. The results of these GWAS is illustrated in Figure 2. The C/T allele at SNP rs9295079, located in an intron towards the 3' end of *SMOC-2* (Figure 3), showed significance and was associated with variability in drug response across individuals (Table 1).<sup>26</sup> *SMOC-2* was thus chosen to be

validated as the gene responsible for the rituximab resistant phenotype since the SNP had the highest significant p-value for rituximab and was found in an intron within this gene.

*SMOC-2* (SPARC Related Modular Calcium Binding 2) encodes a member of the SPARC family, which promotes matrix assembly and can stimulate angiogenic activity as well as endothelial cell proliferation and migration.<sup>28</sup> Several experiments have implicated SPARC, a related protein of SMOC-2, as an indicator of poor prognosis very often associated with the most aggressive tumors in a vast majority of human cancer types. Adenoviral constructs and non-viral plasmids expressing an antisense RNA and a siRNA were used to block *SPARC* expression. In both cases, a strong inhibition or a complete abrogation of human melanoma growth in nude mice was observed.<sup>29</sup> Furthermore, by using antisense RNA to suppress *SPARC*, the motility and invasion of human breast cancer cells *in vitro* were inhibited. Even though there is a significant amount of evidence about the role that SPARC can play in tumor growth, not many experiments have been performed to establish SMOC-2's role in tumor growth or its mechanism of action. Since SPARC is known to have a key dynamic role in increased cancer aggressiveness, SMOC-2 may potentially function in similar ways and thus needs to be assessed.



**Figure 2. Manhattan Plot for Rituximab, Ofatumumab, and Multivariate GWAS.** The negative log transform of p-values for three GWAS are given for 2.1 million SNPs. Nominal p-values are provided for all cases except any significant associations  $-\log_{10}(p) > 6$ ; blue threshold line above. For all significant associations, the p-values are adjusted for multiple comparisons via permutation testing.<sup>26</sup>

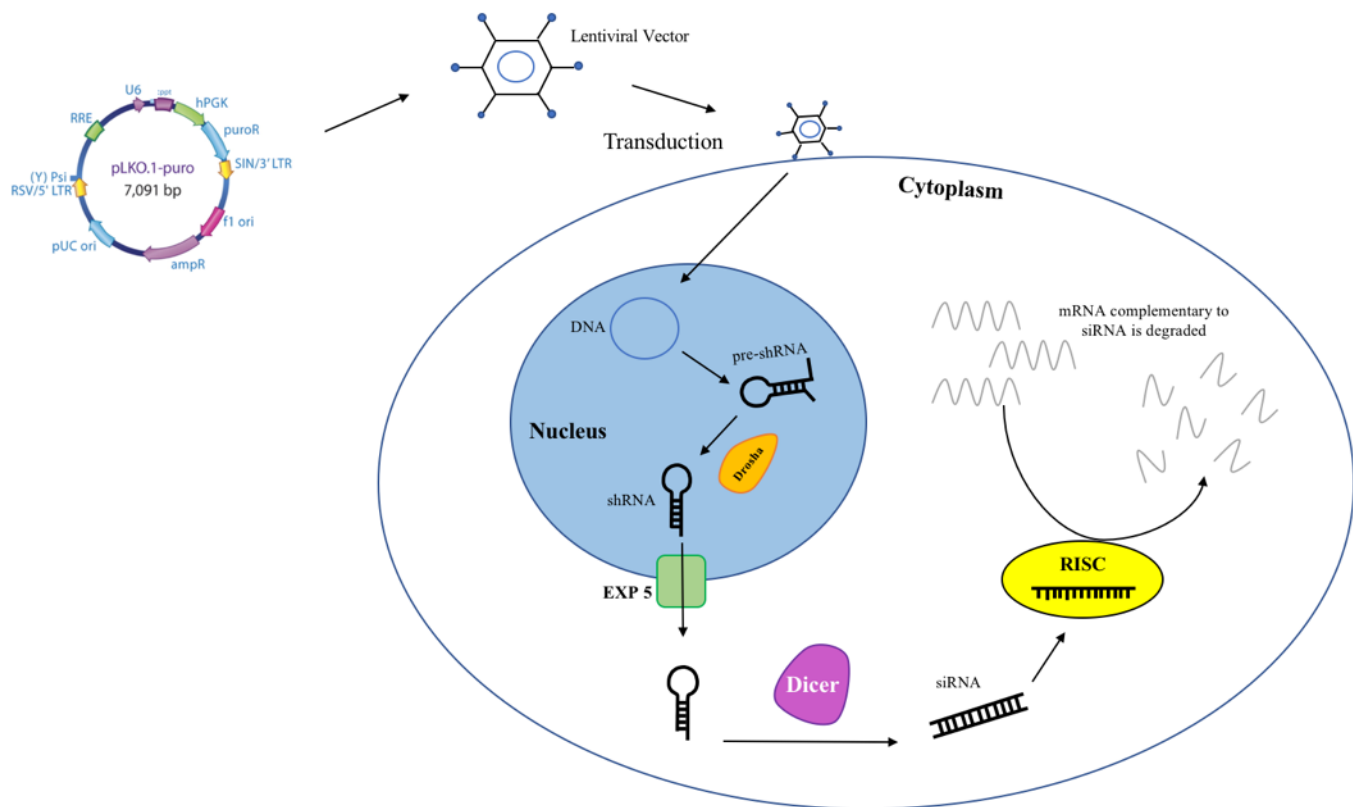
Drug	ChrNum	RSID	nAA	nAa	naa	p-value	$-\log_{10}(p\text{-value})$	Permutation p-value	$-\log_{10}(\text{Permutation p-value})$	Suggestive	Significant	Gene
Rituximab	6	rs9295079	145	247	94	6.38E-007	6.19	7.47E-007	6.13	Yes		SMOC2
Rituximab	18	rs9953394	6	111	369	5.74E-007	6.24	1.34E-006	5.87	No		MIR4318*
Ofatumumab	4	rs356335	370	112	4	2.50E-007	6.60	1.99E-006	5.70	No		None
Ofatumumab	5	rs10070859	407	75	4	3.20E-008	7.49	2.40E-007	6.62	Yes		APC*
Combined	5	rs10070859	407	75	4	1.12E-008	7.95	2.40E-007	6.62	Yes		APC*
* within 100 Kbp of gene encoding region												

**Table 1. Summary of Peak P-values Found in Rituximab and Ofatumumab GWAS.** Chromosome location, ID, genotype distribution, and nearby genes are given for each SNP. Nominal p-values are given along with p-values adjusted for multiple comparisons via permutation testing. Peak associations are marked “Yes” for significance if the negative log transform of the adjusted p-value is greater than 6.<sup>24</sup> The *SMOC-2* gene is implicated to be significant in mediating response to rituximab.<sup>26</sup>



**Figure 3.** C/T allele at SNP rs9295079 maps to a 3' intron of the *SMOC-2* gene (Adapted from NCBI).

A *SMOC-2* knockdown cell line was created to determine this gene's role in mediating a cancerous B-cell's response to rituximab treatment. Gene knockdown was performed using RNA interference via shRNAs introduced with lentiviral transduction, and a stable knockdown cell line was established using puromycin selection (Figure 4). CDC assays were then performed in order to ascertain if there was a change in rituximab sensitivity. Finally, a western blot was utilized to show *SMOC-2* suppression in the knockdown cell line. This study demonstrates how genome-wide mapping can be used to discover and elucidate novel biological mechanisms of potential clinical advantage. Therapies targeting *SMOC-2* could tremendously aid in treating patients with lymphoma who may have acquired or inherited resistance to certain monoclonal antibodies.



**Figure 4.** Gene silencing due to RNAi. After being expressed in the nucleus, Drosha processes the shRNAs, which are then exported to the cytoplasm by Exportin-5. Dicer removes the loop sequence from the shRNA. The shRNAs then associate with RISC, removing one of the RNA strands. The shRNAs, now functionally siRNAs, target complementary mRNA molecules, resulting in their degradation.

## Methods

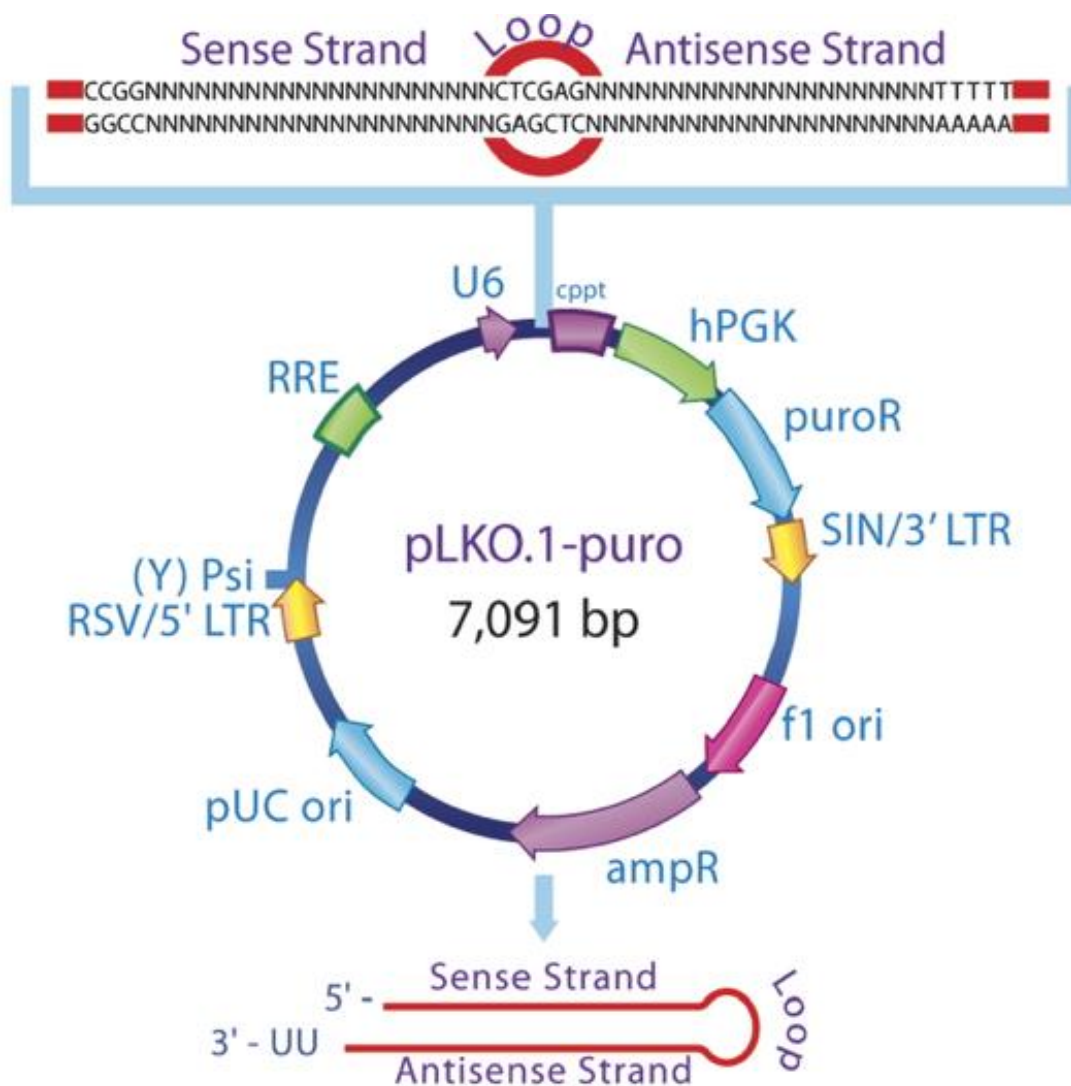
### RNAi Mediated *SMOC-2* Knockdown

Gene knockdown was performed using shRNA (short hairpin RNA) to induce RNA interference (RNAi). RNAi is the process by which expression of a target gene is effectively silenced or knocked down by the selective inactivation of its corresponding mRNA by double-stranded RNA (dsRNA). The Lentihair vector was used to create pLKO.1, which carries the

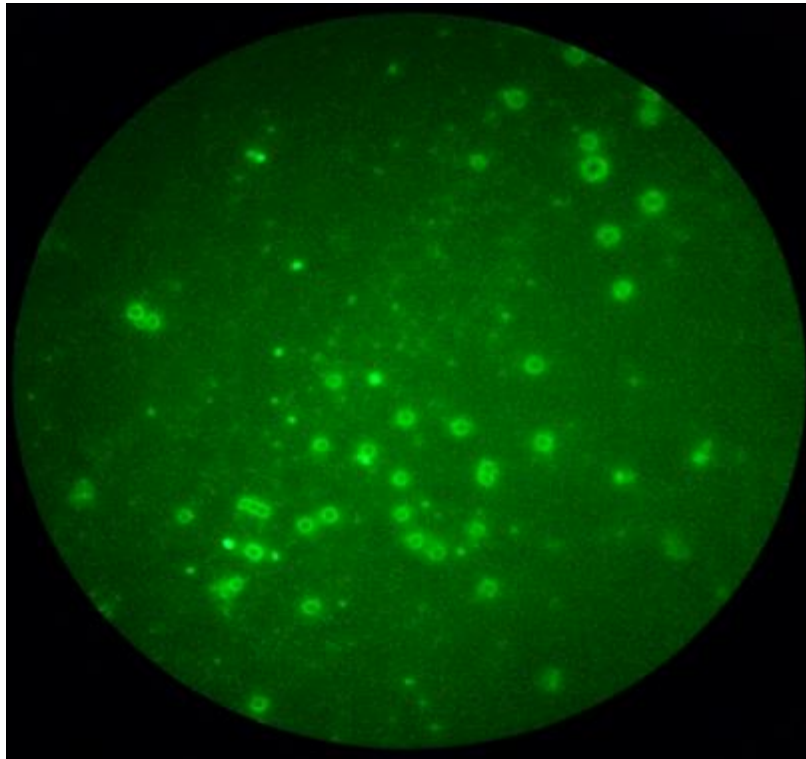
puromycin-resistance gene and drives shRNA expression from a human *U6* promoter (Figure 5).<sup>29</sup> Transient *SMOC-2* knockdown cell lines were prepared by transduction with lentivirus encoding *SMOC-2* specific shRNA sequences. To garner virus, low-passage HEK293T cells were transfected with 5 different pLKO.1 plasmids each encoding a unique *SMOC-2* shRNA. After testing each shRNA knockdown cell line with CDC assays, shRNA 43 showed the strongest phenotypic change, and subsequent transductions utilized this shRNA's viral supernatant. The medium was replaced with fresh media 18 hours after transfection, and viral particles were collected twice at 24-hour intervals. Cells were then transduced with this viral supernatant supplemented with polybrene via spin transduction. After viral supernatant was added to the target cells, the cells were first incubated for 20 minutes and then spun for 30 minutes at 14,000 xg. Cells were subsequently resuspended in RPMI 10% FBS (gibco #11875-093). pLKO served as a vehicle control. Side-by-side GFP transductions were used as a positive control to show that the transduction protocol worked overall and to determine transduction efficiency (Figure 6). Some cells were transduced with the GFP control while other cells of the same cell line were transduced with either the shRNA 43 or control plasmid. GFP fluorescence indicated that about 60% of cells were transduced (Supplementary Figure 2). Stable DLBCL *SMOC-2* knockdown cell lines were established via puromycin selection (1 ug/mL) for 10 days in order to ensure that the plasmids containing shRNA 43 and the puromycin resistance marker were taken up by the cells.

**shRNA 43 sequence:**

CCGGGTGACGTGAATAATGACAAATCTCGAGATTTGTCATTATTCACGTCACCTTTTTG



**Figure 5.** For each shRNA, stem sequences were designed matching a 21-base region of the target transcript with an intervening 6-base “loop” consisting of an XhoI site. Lentiviral vector was used to create pLKO.1, which carries the puromycin-resistance gene and drives shRNA expression from a human *U6* promoter.<sup>29</sup>



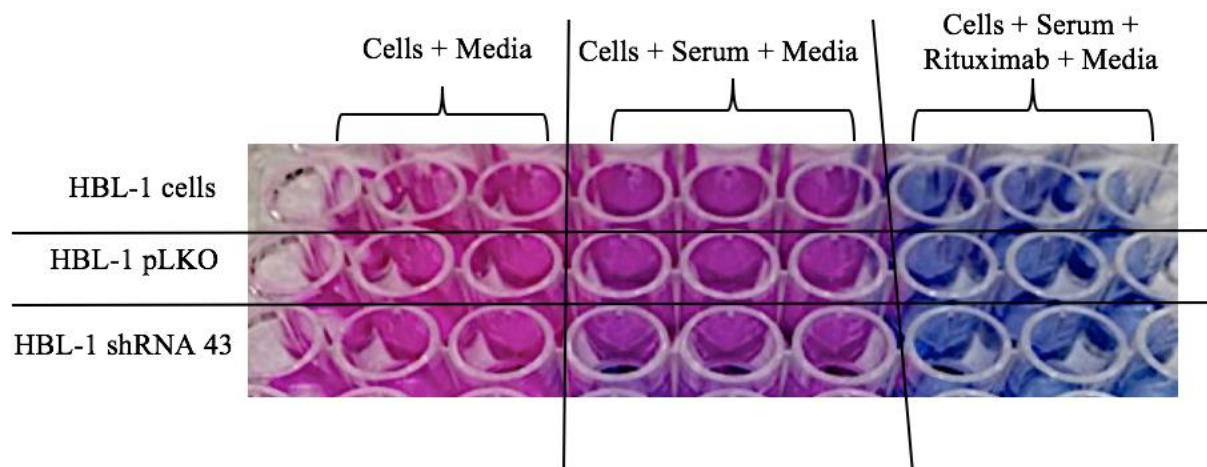
**Figure 6.** GFP Expression of HBL-1 cells indicating about the same transduction efficiency as Raji cells at about 60% (See Supplementary Figure 2).

### **CDC Assay for *SMOC-2***

CDC Assays were used to test whether the *SMOC-2* knockdowns exhibited altered sensitivity to rituximab. HBL-1 *SMOC-2* knockdown cells were plated at  $1 \times 10^4$  cells in a 96-well plate in a total volume of 100  $\mu$ l media containing 25% pooled human serum with or without 1% rituximab at 10  $\mu$ g/ml (Genetech NDC 50242-053-06). Proliferation was then measured using alamarBlue (Thermo #DAL1100). This reagent was added to samples and several time points were taken, including a 24-hour reading. Fluorescence was measured with excitation at 540 nm and



emission at 620 nm on a BioTek™ Synergy™ 2 Multi-Mode Microplate Reader. Proliferation was measured relative to samples without rituximab in order to determine cell viability (Figure 7).



**Figure 7.** CDC Assay Plate set up

### Western Blotting for SMOC-2

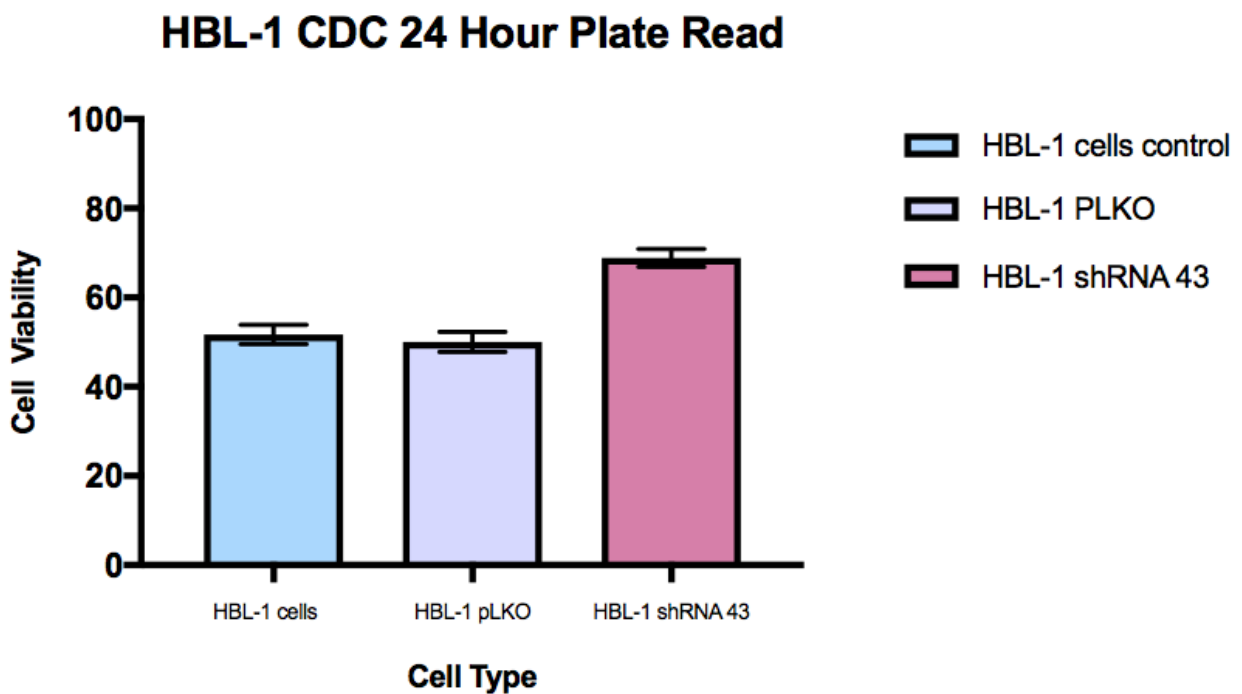
$2 \times 10^6$  cells were lysed in RIPA buffer (Thermo #89901), 1X Halt Protease Inhibitor (Thermo #1861281), and EDTA (Thermo #1861274).<sup>30</sup> An aliquot was used for protein determination using the BCA protein assay kit (#23227). Protein levels were quantified with a BioTek™ Synergy™ 2 Multi-Mode Microplate Reader. The remaining sample was mixed with 1X lamelli buffer (Bio-Rad #161-0747) containing b-mercaptoethanol (Sigma #125K0165) and heated for 5 minutes at 95 °C. Equivalent protein amounts were run on SDS-PAGE in Tris/glycine/SDS buffer using 10% acrylamide gels. Resolved proteins were then transferred to PVDF membranes (Thermo #88518). Blots were blocked using a 3% solution of nonfat dry milk (VWR #M203-10G-10PK) in TBS and Tween 20 (Fisher #074605) and exposed to antibodies in the same buffer. Lysates from a mouse's heart, harvested from a humanely euthanized mouse used

in a separate IACUC-approved study, were used as a positive control for *SMOC-2* expression. The first *SMOC-2* primary antibody used was a rabbit monoclonal antibody (Santa Cruz – 67396) raised against amino acids 71-123 mapping near the N-terminus of *SMOC-2* of human origin (1:200). After this antibody performed rather poorly in terms of specificity, a rabbit polyclonal antibody (Thermo PA5-31892), which recognized a recombinant protein fragment corresponding to a region within amino acids 260 and 396 of Human *SMOC-2*, was obtained and tested (1:1000). Anti- $\beta$ -actin (Sigma #A5441) was used as a load control (1:5000). Anti-rabbit IgG peroxidase secondary antibodies (Cell Signaling #7074S) were used to visualize immunoreactive bands for *SMOC-2* (1:5000) while anti-mouse IgG peroxidase secondary antibodies (Cell Signaling #7076S) were used for beta actin (1:20000). Immobilon<sup>TM</sup> Western Chemiluminescent HRP Substrate (EMD Millipore #WBKLS0500) was used to visualize immunoreactive bands.

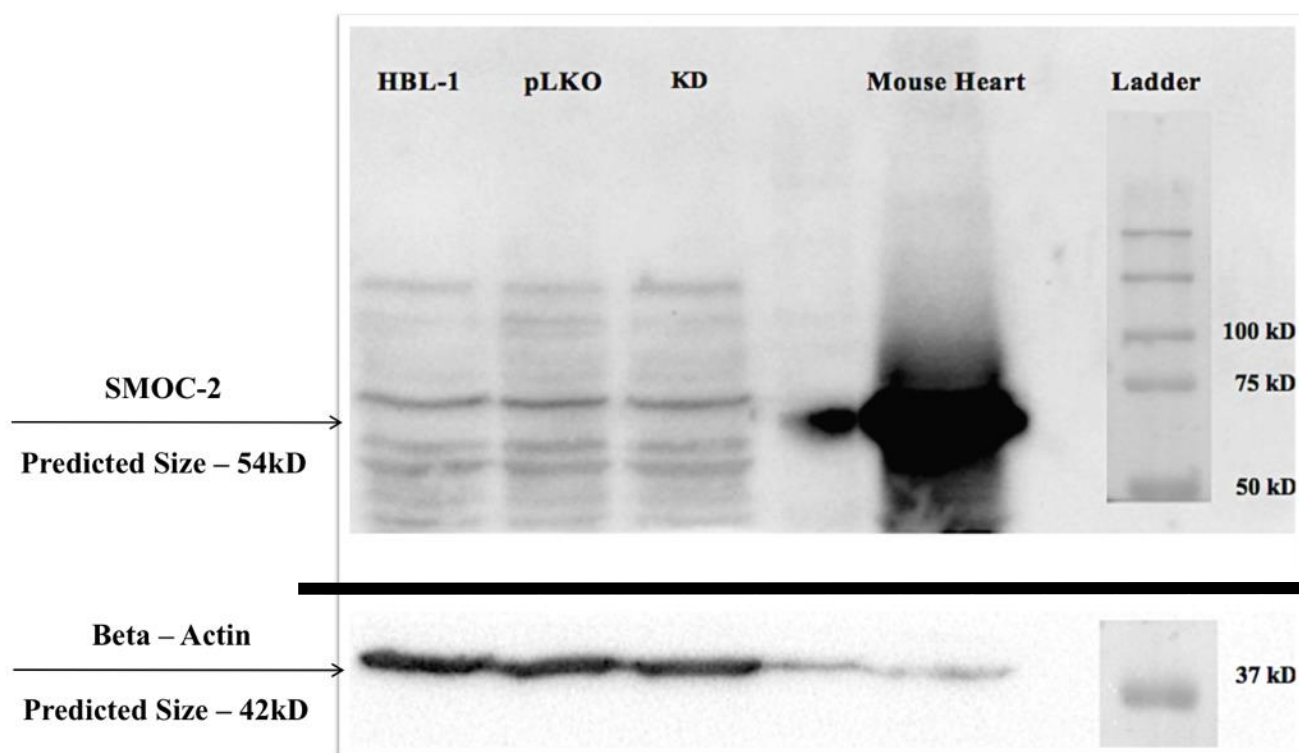
## Results

Successful transductions for *SMOC-2* knockdown cells were marked by green fluorescent cells (Figure 6, Supplemental Figure 2, Supplemental Figure 4). CDC assays showed that *SMOC-2* knockdown causes rituximab resistance in HBL-1 cells since more cells were viable after 24 hours when treated with rituximab (Figure 8, Supplementary Figure 1). The other cell lines transduced with shRNA 43 (Raji and SUDHL-10) did not produce consistent results to draw any conclusions (Supplemental Figure 3 and Supplemental Figure 5). A western blot was initially used to assess the *SMOC-2* knockdown but no visible reduction in *SMOC-2* protein levels in shRNA 43 transduced cells (KD) was observed, possibly due to a poorly specific antibody (Figure 9). Before running a western blot with a newly obtained *SMOC-2* primary antibody, a CDC assay was

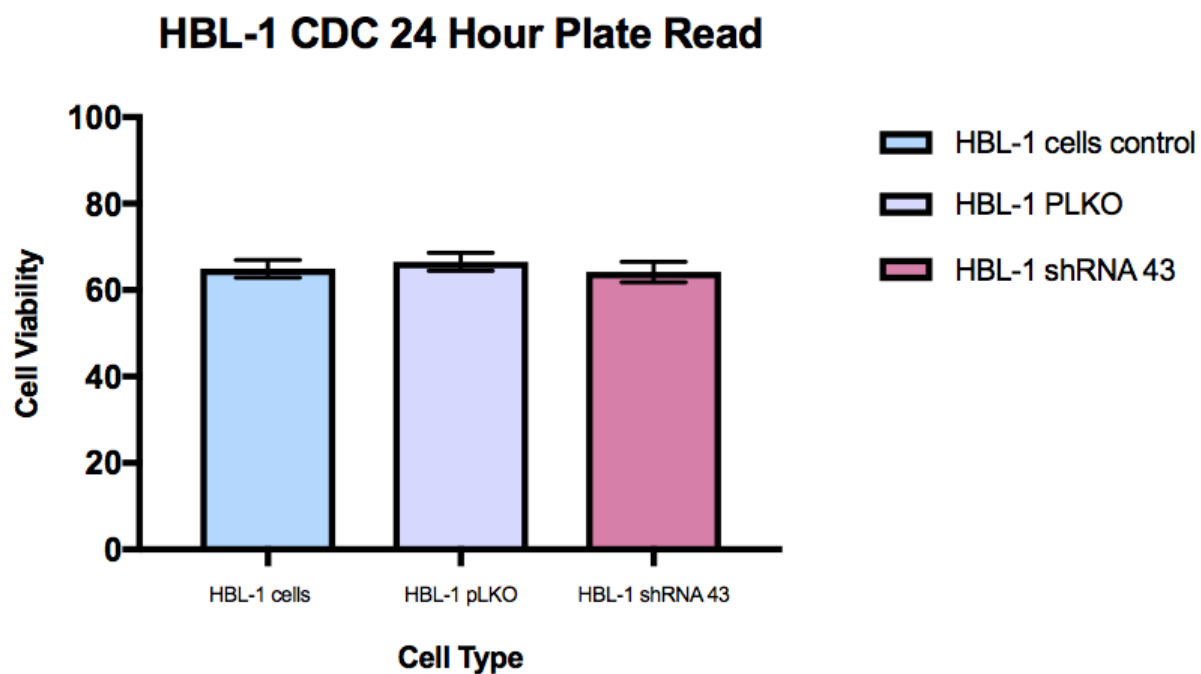
performed on cells that were put back under selection with puromycin (1 ug/mL). The assay revealed no noticeable change in rituximab sensitivity, i.e., the resistant phenotype was not obtained as before. A band for the predicted molecular weight of SMOC-2 was observed in untransduced HBL-1 control cells at 50 kD (predicted SMOC-2 mass in humans = 49.6 kD) (Figure 11). However, the mouse heart lysate, which was used as a positive control, conveyed two bands for SMOC-2, one around 70 kD and the other at 23 kD.



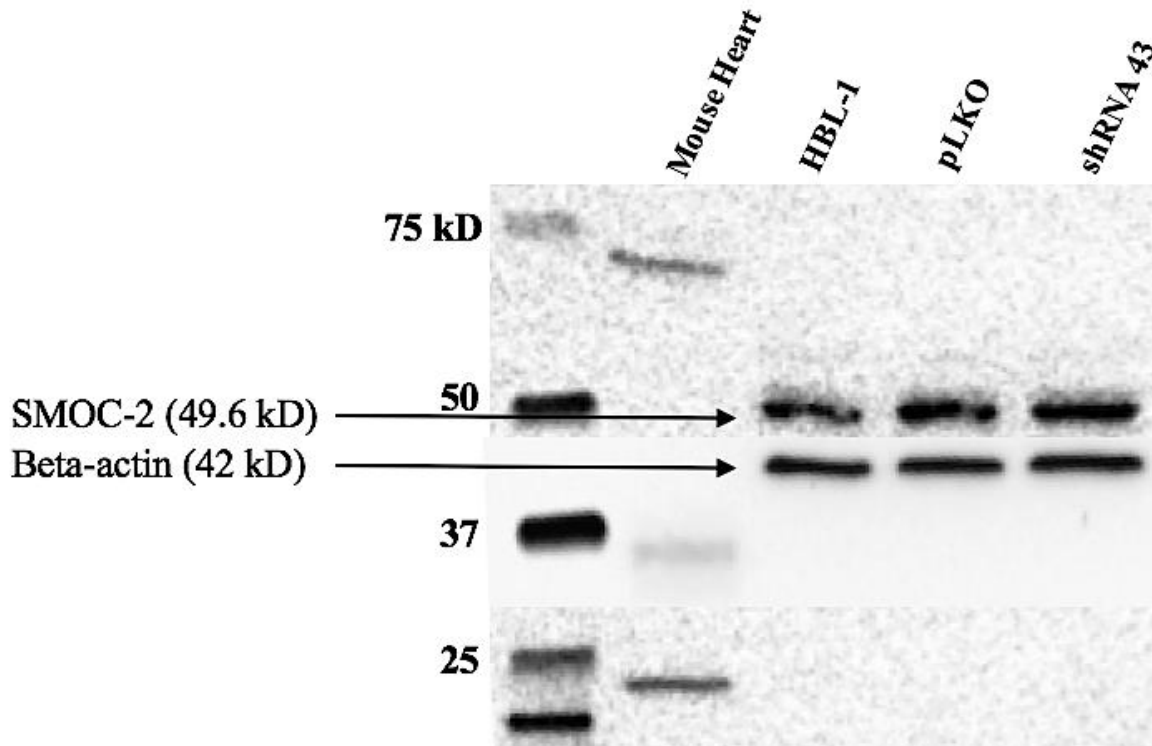
**Figure 8.** CDC Assay results for HBL-1 *SMOC-2* Knockdown Cells 24 hours after incubation with rituximab. Data suggests that cells are resistant to rituximab if the *SMOC-2* gene is suppressed.



**Figure 9.** Western blot for HBL-1 *SMOC-2* Knockdown Cells – Resistant Phenotype. There is no noticeable difference between the HBL-1 and knockdown (KD) bands, possibly due to a poorly specific antibody.



**Figure 10.** CDC Assay results for HBL-1 *SMOC-2* Knockdowns. Data suggests that sensitivity to rituximab may not be altered. *SMOC-2* knockdown must be assessed before drawing conclusions.



**Figure 11.** Western blot for HBL-1 *SMOC-2* Knockdown Cells using a different primary antibody. There is no noticeable difference between the HBL-1 control and knockdown (shRNA 43) bands. However, the antibody conveyed different band sizes for mouse and human *SMOC-2*, which means we cannot conclude if the bands at ~50 kD are actually the *SMOC-2* protein.

## Discussion

This study demonstrates the ability of genome-wide mapping to uncover the potential clinical advantages pertaining to novel biological mechanisms. The transduction protocol utilized in this study worked well to introduce plasmids containing shRNAs targeted towards the *SMOC-2* gene. GFP control cells were viewed under the microscope for green fluorescence as an indirect measure of shRNA transduction efficiency. Initial CDC assays indicated that *SMOC-2* knockdown caused HBL-1 cells to become resistant to the rituximab antibody since there was higher cell viability after 24 hours for the shRNA 43 cells treated with rituximab. The other cell lines transduced with shRNA 43 (Raji and SUDHL-10) did not produce sufficiently consistent results

to draw any conclusions. The Raji cell line did not display any changes to rituximab sensitivity. The SUDHL-10 cell line was resistant to rituximab at baseline, so detecting an additional significant increase would be difficult. A western blot was used to assess the *SMOC-2* knockdown but no visible reduction in *SMOC-2* protein levels in shRNA 43 transduced cells was observed. Although the western blot was inconclusive, the result is likely due to a lack of specificity of the antibody. A new *SMOC-2* primary antibody was then acquired. Cells were placed back under puromycin selection before lysates were obtained. CDC assays, however, revealed that these *SMOC-2* knockdown cells did not display the rituximab resistant phenotype as initial CDC results had shown. The stress that the knockdown cells encountered when undergoing freezing and thawing as well as repeated exposure to puromycin may have caused the loss of the *SMOC-2* knockdown. A western blot using the new *SMOC-2* primary antibody showed bands at the predicted molecular weight for *SMOC-2* in humans (49.6 kD). However, *SMOC-2* in the mouse heart sample showed two bands that were not near the predicted molecular weight in mice (49.9 kD). The band at ~70 kD could have ran higher due to post-translational modifications to the *SMOC-2* protein, but 20 kD of additions/alterations is suspect. Thus, a definitive conclusion about the suppression of *SMOC-2* in the knockdown cell lines could not be established due to the difficulties in distinguishing the *SMOC-2* band in the western blots. These outcomes indicate that there is a possibility that *SMOC-2* suppression may lead to resistance to rituximab but the *SMOC-2* knockdown needs to be evaluated before a decision can be made. RNA data by Sadia Salahud Din conveys that LY10 cells have low expression of *SMOC-2* while 11828 cells show high expression. LY10 will be used as a negative control, and 11828 will be used as a positive control in the next western blot (Supplementary Figure 6).

Since SMOC-2 is an extracellular matrix protein, further studies into how the CD-20 receptor is altered when *SMOC-2* is suppressed may lend support to the above findings. To determine whether absence of SMOC-2 alters CD20 expression, total cellular protein levels of CD20 could be measured. To further examine CD20 localization as a possible mechanism for the effects of reduced SMOC-2 levels, immunofluorescence, where CD20 receptors are tagged, could be performed in *SMOC-2* knockdown cells. The full mechanism through which *SMOC-2* expression leads to altered anti-CD20 antibody susceptibility will require additional studies. The applicability of our results to patients receiving rituximab remains to be determined. In general, there are obvious immunologic and systemic features lacking in *in vitro* systems, which would affect the fate and survival of B cells *in vivo*. In this study, we investigated one type of cell death by monoclonal antibodies, i.e., complement-dependent cytotoxicity, but monoclonal antibodies also function through apoptotic and antibody-dependent cell death mechanisms. ADCC and apoptotic direct killing assays could also be used to implicate *SMOC-2* in rituximab resistance. If these mechanisms of cell death are also affected by altering SMOC-2 levels and lead to rituximab becoming less efficient in killing cancerous B cells, we could confidently claim that the *SMOC-2* gene plays a pivotal role in regulating cellular responses to rituximab. Understanding this function of SMOC-2 in monoclonal antibody treatment could potentially be used in determining more logical and cost-efficient treatments to combat lymphoma.

Eventually, we hope to be able to use patient genotypes (including at the *SMOC-2* polymorphism) to determine which anti-CD20 antibody would be most effective or find ways to restore rituximab sensitivity in patients who have inherited or acquired resistance. For example, if a particular SNP has been implicated in rituximab resistance, such as the C/T allele at rs9295079, and is associated with a patient, then the patient will receive another drug, such as the monoclonal

antibody ofatumumab or obinutuzumab. In order to determine which drug to give rituximab resistant patients with the *SMOC-2* SNP, CDC and ADCC assays, where a different monoclonal antibody is substituted for rituximab (Supplementary Figure 6), could be performed on cell lines with the known SNP marker, and cell viability would be able to tell us if a certain drug is a better candidate for the patient.

By using SNPs in Genome Wide Association Studies as potential markers for genes that may alter sensitivity to chemotherapeutic drugs, we would be able to uncover more about the mechanisms by which these drugs kill cancer cells. In addition, by elucidating such mechanisms, we could tailor patients' treatment regimens in a more personal manner. This would allow therapies to be both time- and cost-effective for the physician and patient. Chemotherapeutics would not be squandered on patients harboring resistance mutations for certain drugs. Patients would be able to be treated sooner as time would not be wasted on prescribing ineffective drugs for initial treatment. Going forward, personalized medicine may also involve the introduction of new combination therapies or novel cancer drugs. Currently, the National Comprehensive Cancer Network Treatment guidelines recommends a rituximab-based regimen for patients with B-cell lymphoma as the initial therapy.<sup>31</sup> Rituximab resistance, as previously described, often develops within the context of generalized chemotherapy resistance. Through the use of biomarkers, such as SNPs, we can identify whether patients harbor resistance mechanisms to common chemotherapeutics. Given the myriad of rituximab resistance mechanisms that presently exist in addition to those that may arise, innovative treatments are needed for this rituximab-resistant patient population. Radioactive anti-CD20 antibody therapy has also been approved for use in relapsed or refractory low-grade or transformed lymphoma. In a randomized trial, Zevalin, a treatment involving yttrium-90 bound to the murine antibody parent of rituximab and is



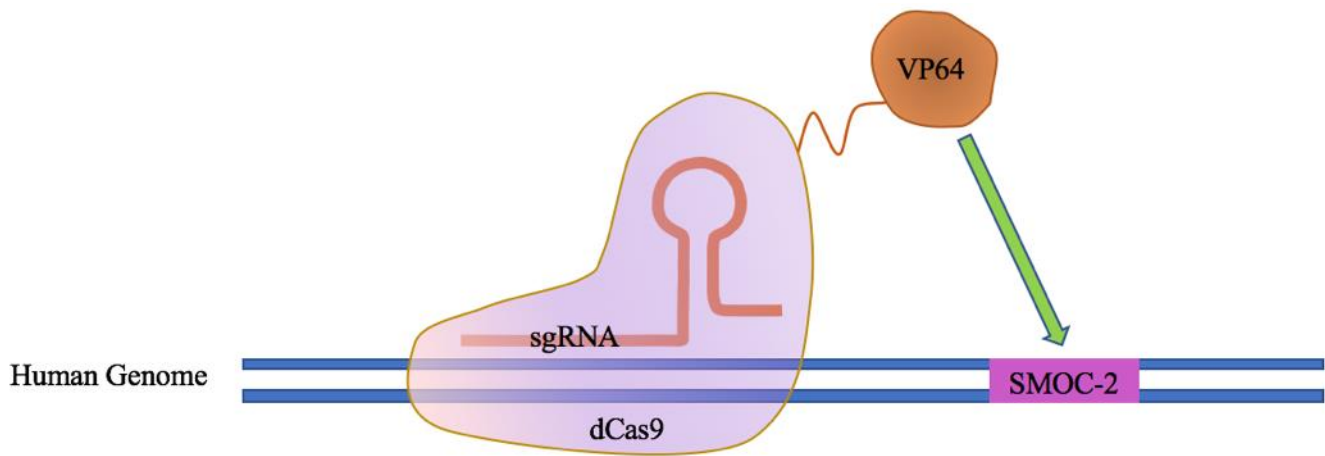
administered along with rituximab to help saturate blood and spleen CD20 sites, had a higher rate of overall and complete responses compared with rituximab alone.<sup>32</sup> A monoclonal antibody similar to rituximab is ofatumumab, which targets an epitope containing the membrane-proximal small-loop on the CD20 receptor. *In vitro* studies involving ofatumumab have demonstrated that this antibody can effectively lyse CLL cells and B cells, especially those displaying low CD20 receptor numbers, better than rituximab.<sup>33</sup> Another monoclonal antibody, obinutuzumab, has been engineered to increase the binding efficiency of effector immune cells, ultimately creating a more efficacious response compared to rituximab. These modifications involve the overexpression of two glycosylation enzymes, MGAT III and golgi mannosidase II, which create antibodies that contain bisecting N-acetylglucosamine and nonfucosylated sugars.<sup>34</sup> By comparing responses to these treatments in rituximab resistant patients, we can further clarify and pinpoint the mechanisms of action of rituximab. Moreover, the discovery of resistance mechanisms for cancer therapeutics, such as rituximab, will pave the way for research into new therapies specifically tailored for individualized patient care.

## **Future Studies**

### ***SMOC-2* Overexpression via CRISPR Activation**

*SMOC-2* overexpression will be accomplished via CRISPR through the use of effectors, i.e., transcriptional activators, fused to deactivated Cas9. The dCas9-VP64 system contains the VP64 transcriptional activator, which is an engineered tetramer of the herpes simplex VP16 transcriptional activator domain (Figure 10). *SMOC-2* knockdown cells may exhibit resistance to

rituximab while *SMOC-2* overexpression cells should in theory exhibit the opposite phenotype: rituximab sensitivity.



**Figure 10.** *SMOC-2* overexpression accomplished via CRISPRa through the use of the VP64 transcription activator, fused to deactivated Cas9. The guide RNA is targeted upstream of the gene.

Forward and Reverse oligonucleotides were designed using Horlbeck et al.'s genome library where a comprehensive algorithm combined chromatin, position, and sequence features to accurately predict highly effective single guide RNAs (sgRNAs) for targeting nuclease-deactivated Cas9-mediated transcriptional repression (CRISPRi) and activation (CRISPRa).<sup>35</sup> Negative controls were also included, which were random sgRNA protospacer sequences generated to reflect the composition of the target sgRNAs and then filtered for sgRNAs with 0 alignments. The guide RNA vectors were cut using BsmBI and forward and reverse guide RNA oligonucleotides were annealed (Figure 10). After ligation with digested pLKO.5 sgRNA EFS GFP vectors, the ligation reaction was incubated overnight and then transformed into competent cells. After heat shock and another overnight incubation, a single colony was selected and QIAGEN spin miniprep was performed. High quality dCas9 plasmid DNA was obtained via

QIAGEN Plasmid midiprep (Figure 11). HEK293FT cells were then plated  $2.5 \times 10^6$  cells in 10% FBS DMEM. After the cells reached 70-80% confluency, DMEM serum free media (gibco #11965-092) was added to Trans-IT (Mirus #2300) along with GAG-Pol, REV, VSV-G, and backbone plasmids. These combination plasmids were added directly to cells. After 12 hours, the old media was removed and replaced by DMEM 10% FBS. Subsequent media containing virus at 36, 72 and 96 hours after initial transfection were collected and pooled. The pooled viral supernatant was centrifuged, and PEG-it concentrator (Systembio #LV810A-1) was added to viral supernatant. After a 12-hour incubation, the supernatant was centrifuged again and resuspended in PBS (Cell Signaling #9808S). This viral procedure was done for both the dCas9 plasmid and guide plasmid. DLBCLs were then transduced by adding 50ul of concentrated virus to 500,000 cells for 12-16 hours. Cells transduced with the dCas9 virus were sorted with flow and then subjected to transduction with the gRNA virus (Figure 11). Green cells were thus identified as cells expressing both the dCas9 and gRNA plasmid at this point (Figure 12). The forward and reverse primers used in this study were:

## **RANK 1**

Forward oligo: 5' CACCGAGAGTGC~~CGCGGGGAAGG~~ 3'  
Reverse oligo: 5' AAAC~~CCTTCCCCGCGGCACTCTC~~ 3'

## **RANK 2**

Forward oligo: 5' CACCGTCTGGG~~CCGCAGCGGGCAC~~ 3'  
Reverse oligo: 5' AAAC~~GTGCCCCGCTGCGGCCAGAC~~ 3'

## **NEGATIVE CONTROL**

Forward oligo: 5' CCACGTGTCGTGATGCGTAGACGG 3'  
Reverse oligo: 5' AAAC~~CCGTCTACGCATCACGACAC~~ 3'

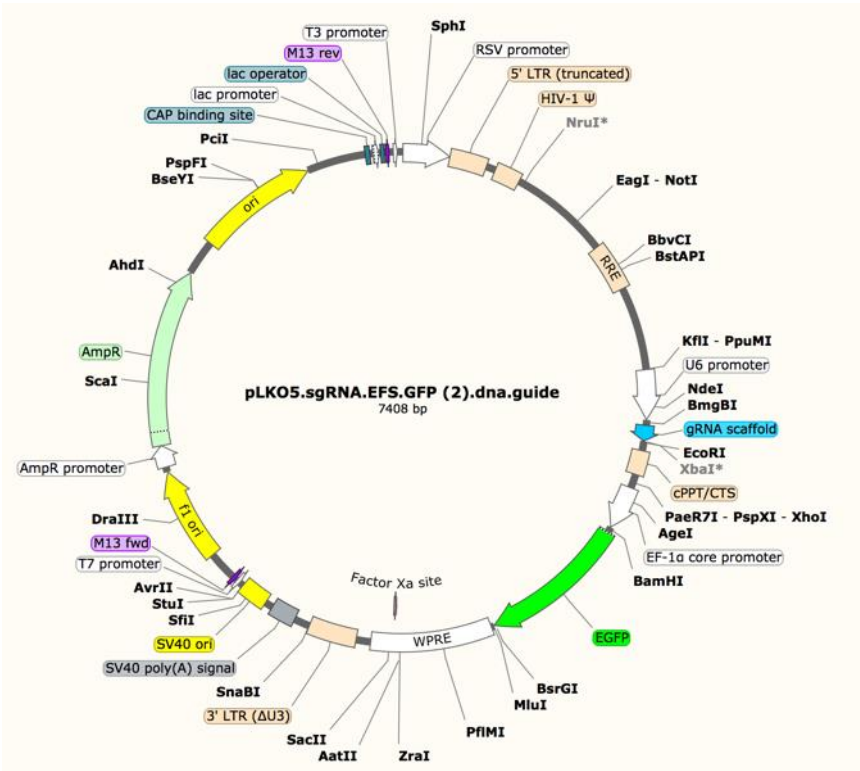


Figure 11. gRNA vector

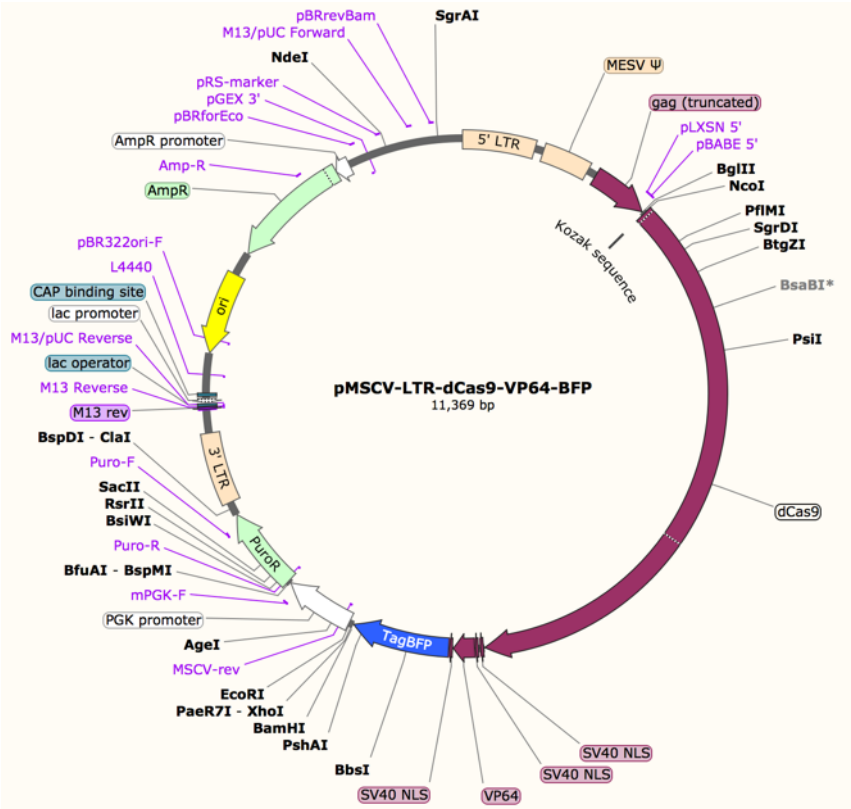


Figure 12. dCas9 vector

*SMOC-2* overexpression should lead to increased sensitivity to rituximab. The transduction protocol utilized in this study for overexpressing *SMOC-2* was able to introduce plasmids containing dCas9. HBL-1 cells were first transduced with the dCas9 plasmid via viral supernatant, and blue fluorescent cells, which indicated expression of the dCas9 plasmid, were observed. However, there was not a high transduction efficiency and thus a distinct blue fluorescent colony could not be flow sorted. Spin transduction will be utilized to correct this problem. In this method, target cells and viral supernatant are incubated together and spun at high speeds (as was performed for *SMOC-2* knockdown). After the dCas9 transduced cells are flow sorted, these cells will be transduced with the gRNA plasmids, and green fluorescent cells would then indicate expression of these guide plasmids. CDC assays will then be performed on *SMOC-2* overexpression cells to determine whether or not these cells are more sensitive to the rituximab antibody. Either a western blot or qPCR can be used to assess if *SMOC-2* overexpression is achieved. These overexpression results combined with the knockdown study would indicate that *SMOC-2* does play an important role in facilitating resistance to rituximab.

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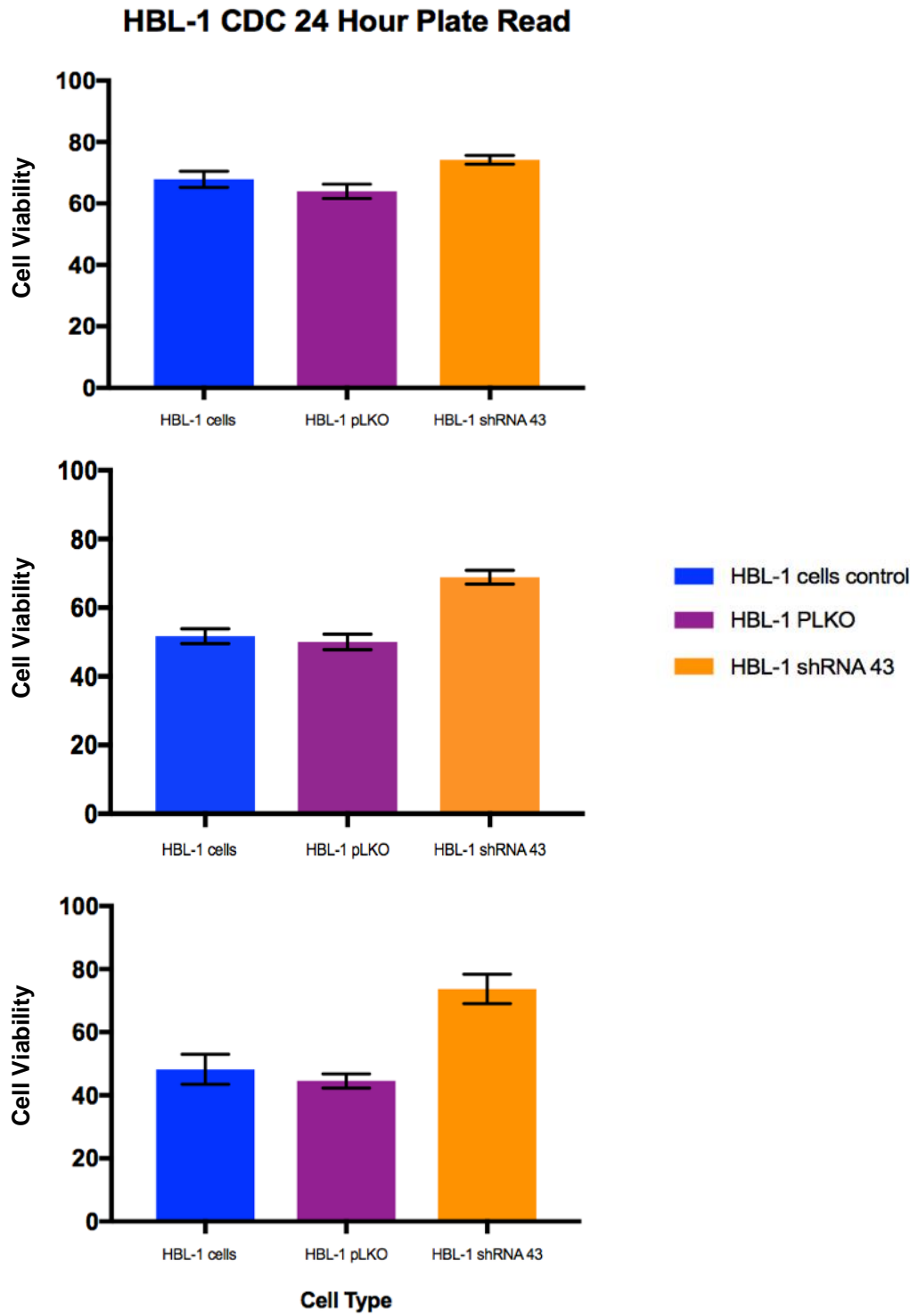


**Acknowledgements**

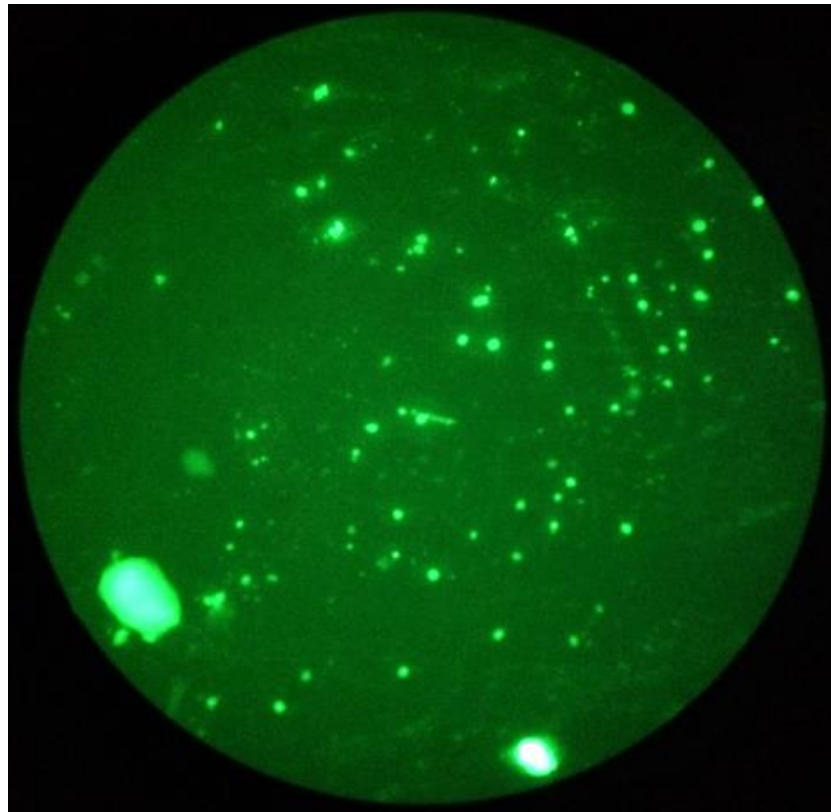
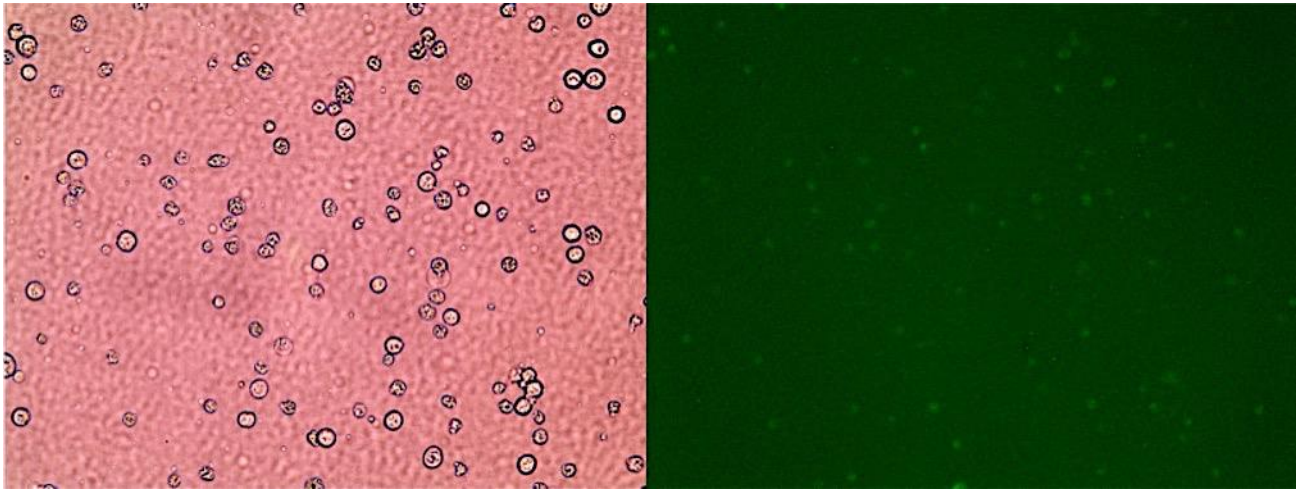
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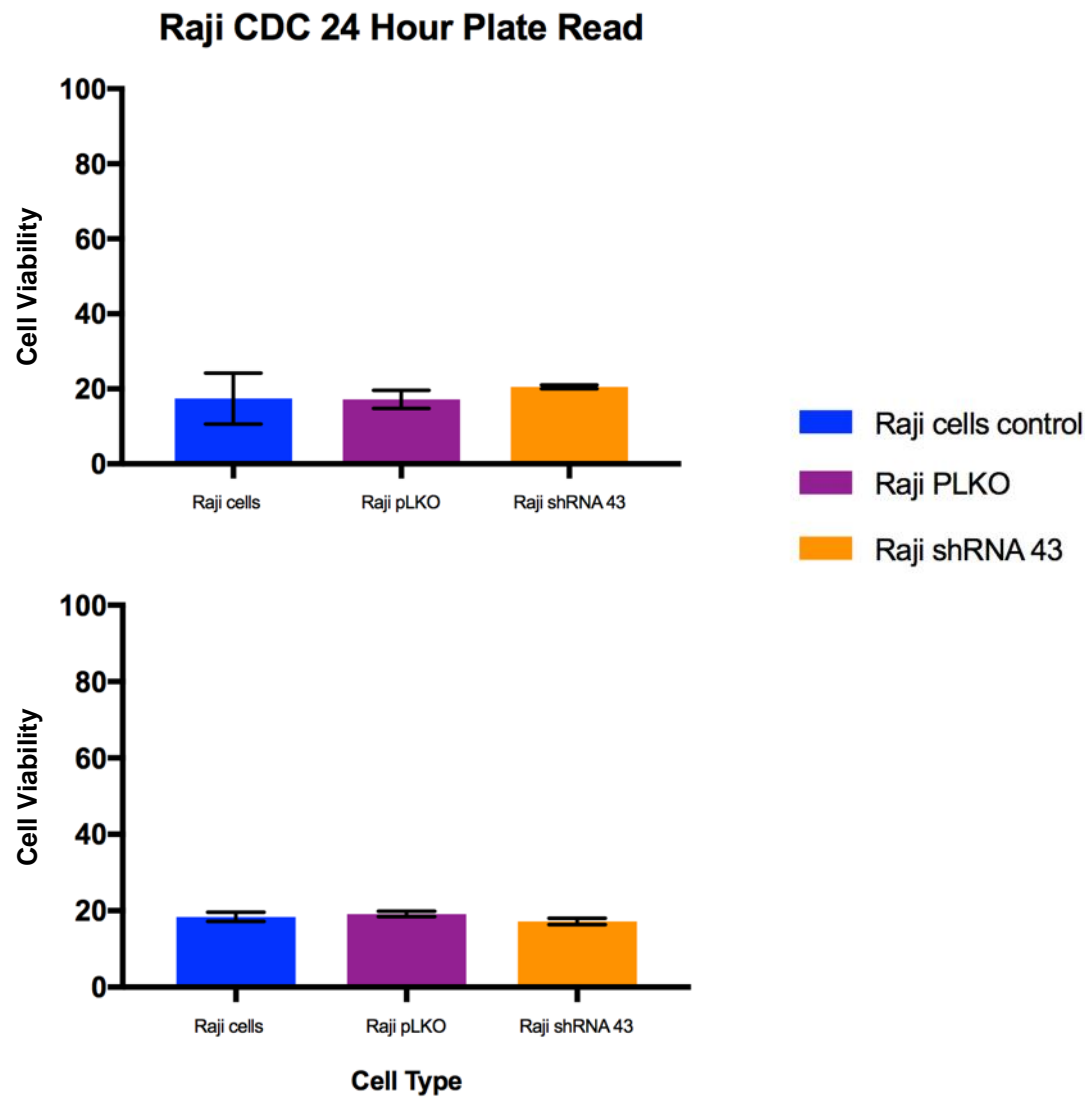
SUPPLEMENTARY FIGURES



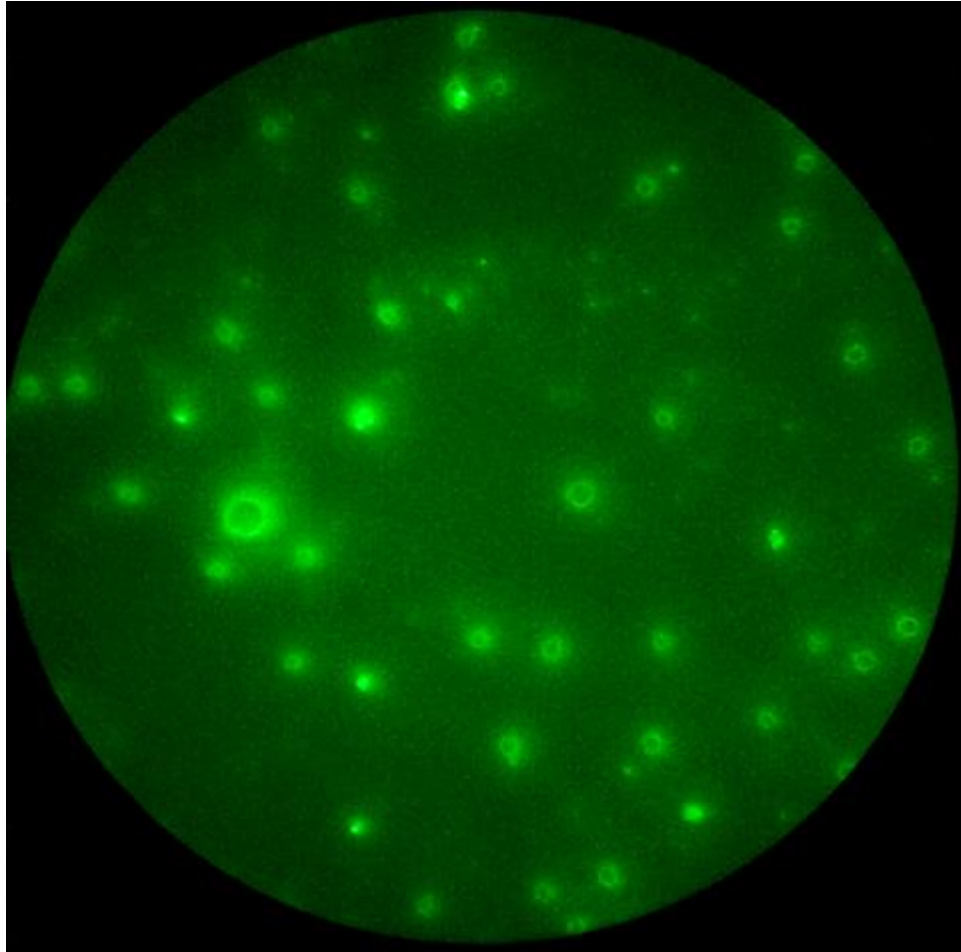
**Supplementary Figure 1.** CDC Assay results for HBL-1 *SMOC-2* Knockdowns 24 hours after incubation with rituximab. Data suggests that HBL-1 cells are resistant to rituximab if the *SMOC-2* gene is suppressed.



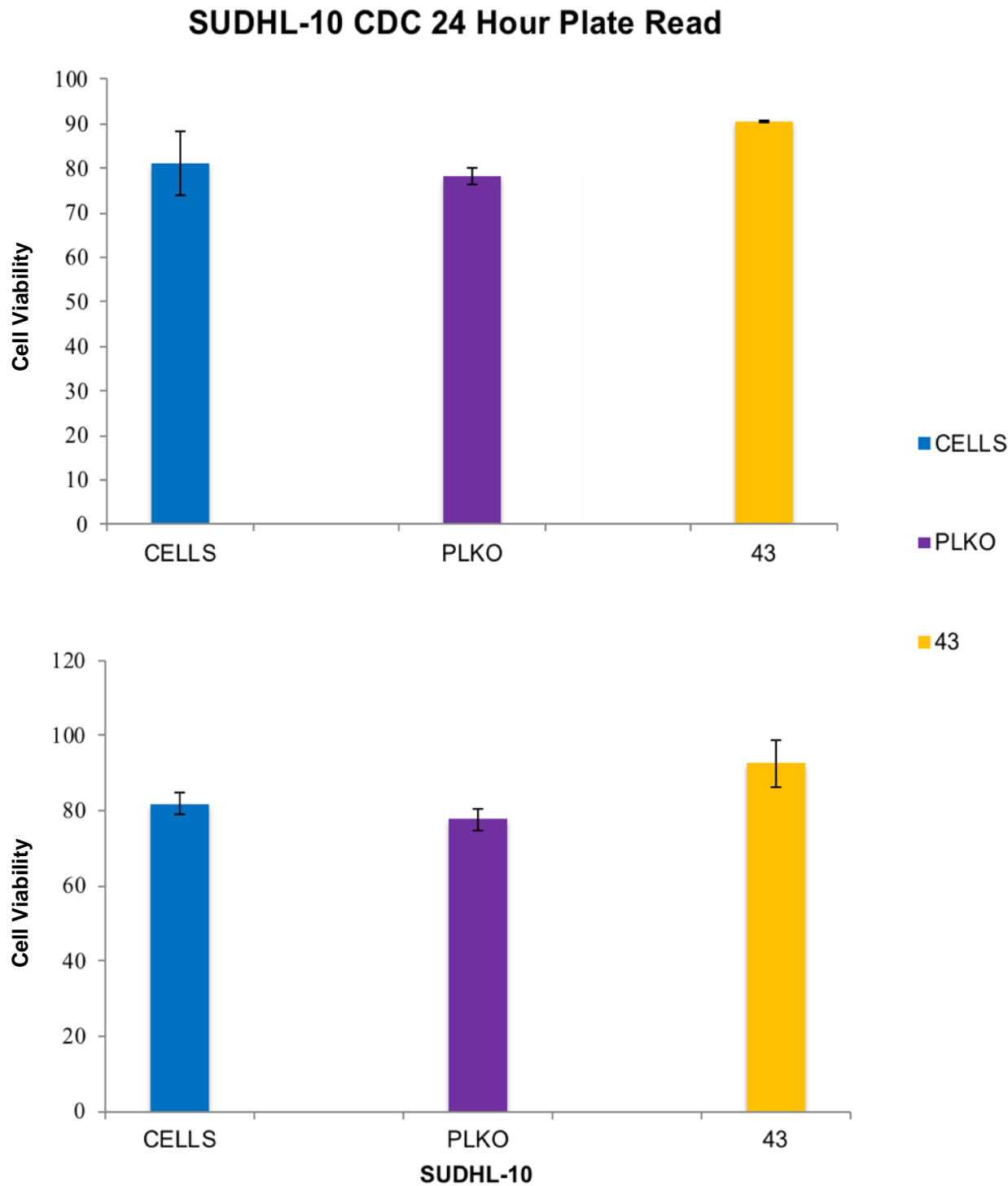
**Supplementary Figure 2.** GFP Expression of Raji Cells indicating a transduction efficiency of about ~60%.



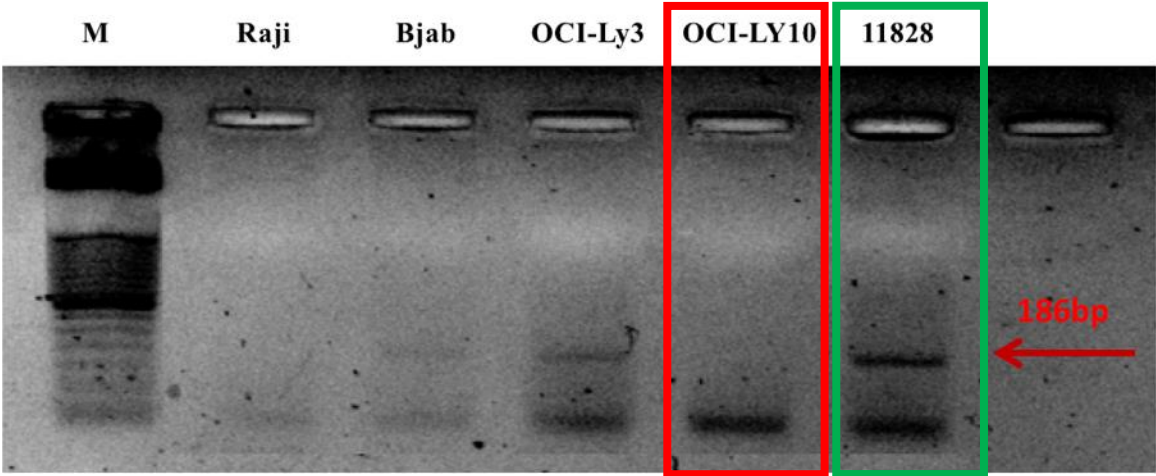
**Supplementary Figure 3.** CDC Assay results for Raji *SMOC-2* Knockdowns 24 hours after incubation with rituximab. Data suggests that Raji cells do not exhibit a change in rituximab sensitivity if the *SMOC-2* gene is suppressed.



**Supplementary Figure 4.** GFP Expression of SUDHL-10 cells indicating successful transduction.

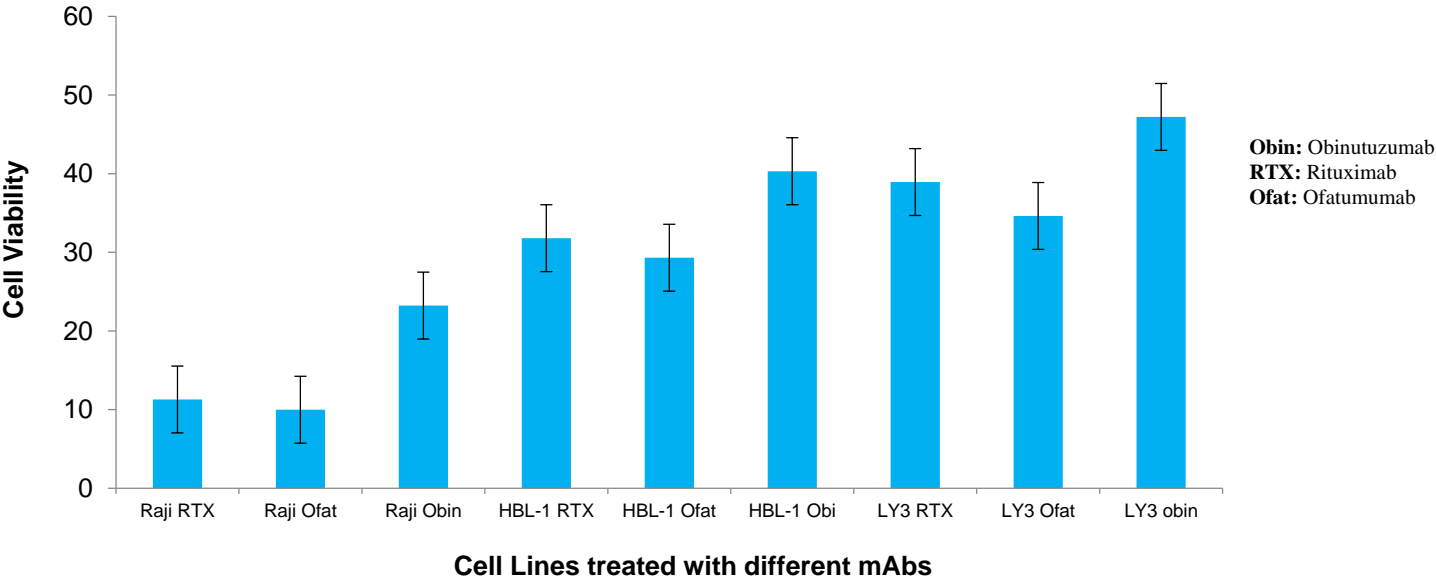


**Supplementary Figure 5.** CDC Assay results for SUDHL-10 *SMOC-2* Knockdowns 24 hours after incubation with rituximab. Cell viability around 80% is difficult to use as a predictor of whether SUDHL-10 cells are resistant to rituximab if the *SMOC-2* gene is suppressed. SUDHL-10 seem resistant to begin with so any added resistance would be challenging to assess.



**Supplementary Figure 6.** RNA data shows low expression of *SMOC-2* in LY10 cells but high expression in 11828 cells. Lysates from 11828 will be used as a positive control while lysates from LY10 will be used as a negative control to discern if the bands ~50 kD are in fact *SMOC-2*. **Courtesy of Sadia Salahud Din**

### CDC 24 Hour Plate Read



**Supplementary Figure 7.** CDC Assay results for Raji, HBL-1, and LY3 cells 24 hours after incubation with rituximab, obinutuzumab, or ofatumumab. **Courtesy of Sadia Salahud Din**